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Auxin-induced Ethylene Production and Its Inhibition by Aminoethoxyvinylglycine and Cobalt Ion[1]

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ABSTRACT

Auxin is known to stimulate greatly both C_2H_4 production and the conversion of methionine to ethylene in vegetative tissues, while aminoethoxyvinylglycine (AVG) or Co^{2+} ion effectively block these processes. To identify the step in the ethylene biosynthetic pathway at which indoleacetic acid (IAA) and AVG exert their effects, [3-^{14}C]methionine was administered to IAA or IAA-plus-AVG-treated mung bean hypocotyls, and the conversion of methionine to *S*-adenosylmethionine (SAM), 1-aminocyclopropane-1-carboxylic acid (ACC), and C_2H_4 was studied. The conversion of methionine to SAM was unaffected by treatment with IAA or IAA plus AVG, but active conversion of methionine to ACC was found only in tissues which were treated with IAA and which were actively producing ethylene. AVG treatment abolished both the conversion of methionine to ACC and ethylene production. These results suggest that in the ethylene biosynthetic pathway (methionine → SAM → ACC → C_2H_4) IAA stimulates C_2H_4 production by inducing the synthesis or activation of ACC synthase, which catalyzes the conversion of SAM to ACC. Indeed, ACC synthase activity was detected only in IAA-treated tissues and its activity was completely inhibited by AVG. This conclusion was supported by the observation that endogenous ACC accumulated after IAA treatment, and that this accumulation was completely eliminated by AVG treatment. The characteristics of Co^{2+} inhibition of IAA-dependent and ACC-dependent ethylene production were similar. The data indicate that Co^{2+} exerts its effect by inhibiting the conversion of ACC to ethylene. This conclusion was further supported by the observation that when Co^{2+} was administered to IAA-treated tissues, endogenous ACC accumulated while ethylene production declined.

Auxins are known to stimulate ethylene production in a wide variety of plant tissues (1, 4, 7, 8, 13, 18, 25). Many of the effects of auxin on growth and on other processes, such as epinasty, hook opening, inhibition of growth, root induction, and geotropism, are now attributed to its ability to induce ethylene production (1, 4, 7). In vegetative tissues the rate of ethylene production is thought to be regulated by the internal level of free auxin (1). Cytokinins stimulate ethylene production only slightly, but a remarkable synergistic effect of cytokinins on IAA-induced ethylene production has been observed in etiolated seedlings of several species (4, 8, 12, 13). Auxin-induced ethylene production, as well as other ethylene production systems, are known to be inhibited by AVG[2] (2, 10, 15) and by Co^{2+} (9, 11, 14).

Recently, Adams and Yang (2) have identified ACC as an intermediate in the conversion of methionine to ethylene in apple tissues and have proposed the following biosynthetic sequence: methionine → SAM → ACC → C_2H_4. Although the conversion of methionine to ethylene requires the presence of auxin (5, 20), we have observed that the conversion of ACC to ethylene is not dependent upon IAA (23). We have proposed, therefore, that IAA exerts its effect by inducing synthesis of the enzyme responsible for the conversion of SAM to ACC. In this communication we present data from *in vivo* and *in vitro* studies showing that IAA exerts its stimulatory effect on ethylene production by enhancing the conversion of SAM to ACC and causing the accumulation of ACC. In addition we have shown that AVG inhibits ethylene production by interfering with the conversion of SAM to ACC, and that Co^{2+} ion interferes with the conversion of ACC to ethylene.

MATERIALS AND METHODS

Plant Material. Seeds of mung bean (*Vigna radiata* [L.] Wilczek) were germinated and grown in Vermiculite for 3.5 days in darkness at 25 C. Twenty 2-cm-long hypocotyl segments, 1 to 3 cm below the hook, were incubated in 5 ml of a medium consisting of 2% sucrose, 50 μg/ml chloramphenicol, and 50 mM Mes buffer (pH 6.1) in a 50-ml Erlenmeyer flask. Where indicated, addenda were 50 μM IAA plus 20 μM TZ (Tables I, II, and III), 50 μM IAA plus 25 μM IPA (Table IV, Figs. 1 and 2), 10 μM AVG, and 0.2 mM ACC. The flasks were flushed with air, sealed with rubber serum caps, and incubated at 27 C. At the indicated time, ethylene production was determined and labeled SAM and ACC, and total ACC were extracted and assayed.

Chemicals. L-[3-^{14}C]Methionine was purchased from Research Products International, IAA and IPA from Sigma, SAM from Boehringer, and ACC from Calbiochem. AVG was a gift from J. P. Scannel (Hoffman-LaRoche). TZ was a gift from Dr. J. Corse (USDA Western Regional Research Center, Berkeley, Calif.).

Determination of Ethylene. A 1-ml gas sample was withdrawn from the head space of the flask with a hypodermic syringe, and ethylene was assayed on a gas chromatograph equipped with an alumina column and a flame ionization detector.

Analysis of ^{14}C-SAM. After incubation for 9 h in a medium containing L-[3-^{14}C]methionine, mung bean hypocotyls were homogenized and extracted with $HClO_4$ at a final concentration of 1 N at room temperature for 1 h. The extract was then centrifuged, and the supernatant was neutralized to pH 4.5 at 0 C by slowly adding solid $NaHCO_3$. The precipitated salt was discarded and the supernatant passed through an ion exchange resin (Bio-Rex 70, H^+ form) column. After washing the column with water until neutral, the [^{14}C]SAM was eluted with 0.1 N HCl (21). The effluent was lyophilized and dissolved in 10% acetic acid. The chemical identification of the radioactive material as SAM was carried out by paper electrophoresis at pH 2.2 (10% acetic acid) with authentic SAM, and by acid hydrolysis to homoserine (21) followed by

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[2] Abbreviations: AVG: aminoethoxyvinylglycine [2-amino-4-(2′-aminoethoxy)-*trans*-3-butenoic acid]; ACC: 1-aminocyclopropane-1-carboxylic acid; SAM: *S*-adenosylmethionine; TZ: *trans*-zeatin; IPA: N^6-(Δ^2-isopentenyl)adenine.

Table I. *Influence of IAA or IAA plus AVG on Conversion of Methionine to SAM by Mung Bean Hypocotyls*

In each flask 20 hypocotyls (weight about 1.4 g) were incubated in 5 ml of medium containing 2 μCi and 40 nmol of L-[3-^{14}C]methionine and other components as listed under "Materials and Methods." In IAA and IAA plus AVG treatments, 50 μM IAA plus 20 μM TZ or 50 μM IAA plus 20 μM TZ plus 10 μM AVG were included, respectively. At the end of 9-h incubation, hypocotyls were homogenized and the radioactivity in SAM determined. The uptake of methionine during the 9-h incubation period was 48, 39, and 37%, respectively, in the three treatments.

Treatment	C_2H_4 Production	*S*-Adenosylmethionine	
	nl/9 h	*nCi*	*% conversion*
Control	3	58	2.9
IAA	1014	42	2.1
IAA + AVG	54	55	2.7

Table II. *Influence of IAA or IAA plus AVG on Conversion of Methionine to Ethylene and to ACC by Mung Bean Hypocotyls*

The incubation media are those described in Table I except that 1.5 μCi and 30 nmol of L-[3-^{14}C]methionine were employed. The uptake of methionine during the 9-h incubation period in these treatments was 25, 31, and 36, respectively.

Treatment	C_2H_4		ACC	
	nmol	*nCi*	*nmol*	*nCi*
Control	0.2	0.3	0.5	0.1
IAA	57	21.0	53	21.2
IAA + AVG	2.5	0.7	0.7	0.1

Table III. *Influence of IAA or IAA plus AVG Treatment on Development of ACC Synthase Activity in Mung Bean Hypocotyls*

ACC synthase isolated from the control or IAA-treated tissues was assayed in the absence or presence of 10 μM AVG.

Treatment	ACC Synthase[a]	
	−AVG	+AVG
Control	<2	<2
IAA	67	<2

[a] pmol of ACC formed in 4.5 h by 0.4 ml of enzyme preparation containing 0.4 mg protein.

Table IV. *Effect of Co^{2+} on Level of Endogenous ACC in Mung Bean Hypocotyls Treated with IAA*

For IAA treatment 50 μM IAA plus 25 μM IPA were employed. Ethylene production was assayed for the period between 6 and 9 h, and ACC was determined at the end of the 9-h incubation period.

Treatments	Ethylene	ACC
	nl	*nmol*
IAA	381	46
IAA + 50 μM Co^{2+}	102	63
IAA + 500 μM Co^{2+}	5	77

paper electrophoresis at pH 2.2. Radioactivity on the paper was detected with a radioscanner. SAM was visualized under UV light, and homoserine by spraying with 2% ninhydrin and heating at 100 C.

Determination of ACC. The hypocotyls were homogenized and extracted with sulfosalicylic acid at a final concentration of 3%. After centrifugation, the extracts were passed through an ion exchange resin (Dowex 50-H^+) column and amino acids including ACC were eluted with 2 N NH_4OH. After concentration, the residues were dissolved in 1 ml water. An aliquot was used for assay of total ACC according to the method of Lizada and Yang (16). The sample to be assayed was placed in a test tube, 1 μmol $HgCl_2$ was added, and the volume was brought to 0.9 ml with water. The reaction vessel was then sealed with a rubber serum cap and kept in ice. Approximately 100 μl of a cold mixture of 5% NaOCl and saturated NaOH (2:1, v/v) was injected and the flask was shaken in the ice bath for 10 min. The ethylene liberated was assayed by gas chromatography. The efficiency of the conversion of ACC to ethylene was determined by adding a known amount of authentic ACC as internal standard to another sample solution which was then degraded to yield ethylene as described above. Calculation of the amount of ACC in the sample was based on the determined conversion efficiency (16). For the analysis of radioactive ACC, an aliquot of the above eluate was mixed with 0.5 μmol of authentic ACC and chromatographed on Whatman 3MM paper using 1-butanol-acetic acid-H_2O (4:1:5, v/v) as developing solvent. Radioactivity on the paper was detected by radioscanner. The radioactive region corresponding to ACC was eluted with water and was degraded to ethylene as outlined above. The ethylene produced was then transferred to an evacuated 25-ml scintillation vial. A 0.5-ml gas sample was withdrawn from the vial for ethylene determination by gas chromatography. The remainder of the ethylene was absorbed by injecting 250 μl of 0.25 M $Hg(ClO_4)_2$ reagent (22) into the vial. The radioactive ethylene thus absorbed was assayed in a liquid scintillation counter after mixing with 10 ml of scintillation solution. The radioactive ACC in the sample was calculated from the following equation: Radioactivity in ACC (nCi) = specific radioactivity of ethylene (nCi/nmol) × 500.

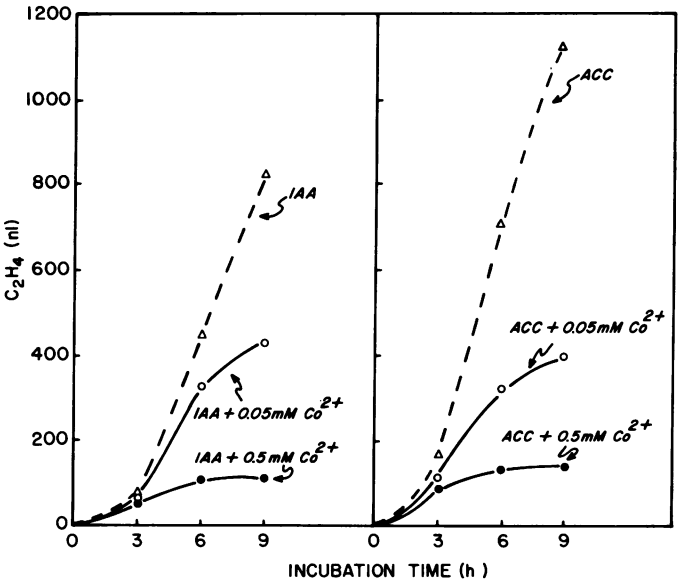

FIG. 1. Effect of Co^{2+} on the time courses of IAA-dependent and ACC-dependent ethylene production by mung bean hypocotyl. The incubation medium for the IAA-dependent system included 50 μM IAA plus 25 μM IPA, and that for the ACC-dependent system included 0.2 mM ACC.

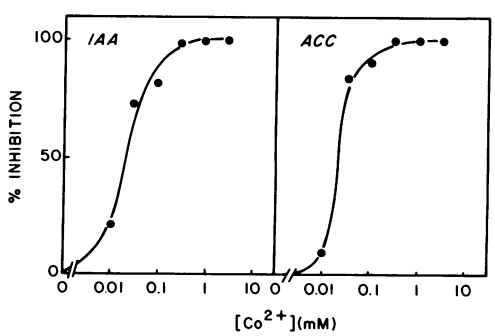

FIG. 2. Effect of concentration of cobalt ion on the inhibition of IAA-dependent and ACC-dependent ethylene production. The incubation media and conditions are as described for Figure 1. Inhibition was calculated for the ethylene produced between 6- and 9-h incubation.

Assay for ACC Synthase. The method used to isolate the enzyme was essentially that described by Boller *et al.* (3). The mung bean hypocotyl tissues were homogenized in Hepes buffer (pH 8.0) containing 0.5 μM pyridoxal phosphate and 4 mM dithioerythritol. The extracts were passed through the Sephadex G-50 column which had been previously equilibrated with 2 mM Hepes (pH 8.0), 0.5 μM pyridoxal phosphate, and 0.1 mM dithioerythritol. The protein fraction was collected and employed as enzyme solution. ACC synthase activity was assayed in a reaction mixture containing 0.4 ml of enzyme, 30 μmol Hepes buffer (pH 8.0), 21 nmol SAM, and 6 nmol AVG, where indicated, in a total volume of 0.6 ml. After incubation at 30 C for 4.5 h, the ACC formed was assayed by the method of Lizada and Yang (16) as described above.

RESULTS AND DISCUSSION

It has been shown that auxin stimulates both ethylene production and the conversion of methionine to ethylene in vegetative tissues (5, 20). The evidence suggests that methionine is a precursor of ethylene and the regulation of auxin-induced ethylene production occurs at a step subsequent to the synthesis of methionine. Since the pathway of ethylene biosynthesis from methionine is now known (2), it is possible to determine at which step in the sequence auxin exerts its hormonal effect. The relative effects of three indicated treatments (control, IAA, IAA plus AVG) upon the incorporation of L-[3-^{14}C]methionine into [^{14}C]SAM and upon the production of ethylene are shown in Table I. Since cytokinins enhance IAA-induced ethylene production (4, 8, 12, 13), cytokinins were included in all of the IAA treatments. Even though IAA stimulated ethylene production about 500 times over that of the control, and AVG at 10 μM completely abolished this IAA-induced ethylene production, the incorporation of label from methionine into SAM was little affected. It is obvious that the conversion of methionine to SAM is not subject to the regulation responsible for either IAA induction or AVG inhibition of ethylene synthesis.

Comparison of the influence of IAA or of IAA plus AVG on ethylene production, conversion of methionine to ethylene, conversion of methionine to ACC, and accumulation of ACC demonstrates a close relationship among these four processes (Table II). Differences in uptake of methionine were slight, and cannot have contributed significantly to differences in the conversion of methionine to ACC and to ethylene observed among these treatments (Table II). Active conversion of methionine to ethylene and to ACC was observed only in tissue which had been treated with auxin and which produced large amounts of ethylene. The specific radioactivity of ethylene was essentially identical to that of ACC (Table II), indicating that all of the ethylene was derived from ACC. In the presence of AVG, IAA-induced ethylene production, conversion of methionine to ethylene, and conversion of methionine to ACC were all abolished.

Since auxin did not influence the conversion of methionine to SAM (Table I) but caused active conversion of methionine to ACC (Table II), auxin must exert its regulatory function on a step involved in the conversion of SAM to ACC. It is known that auxin-induced ethylene production is characterized by a substantial lag period and is vulnerable to inhibitors of RNA and protein synthesis (1, 4, 13, 20). These characteristics are interpreted to indicate that auxin-induced ethylene production requires synthesis of a new enzyme (1, 4). Such a view leads to the proposal that auxin induces ethylene production by inducing the synthesis of ACC synthase. Indeed, ACC synthase activity was found only in the tissue treated with IAA (Table III). ACC synthase has been recently demonstrated by Boller *et al.* (3) in extract of tomato fruits. As predicted from the *in vivo* study by Adams and Yang (2) with apple tissue, ACC synthase utilizes SAM specifically as its substrate (3, 24). An increase in ACC synthase activity accounts for the increase in endogenous ACC level. The effect of IAA and IAA plus AVG treatments on endogenous ACC levels is shown in Table II. IAA treatment increased the ACC level 100 times over that of the control. The limited quantity of ACC in the control tissue obviously restricted ethylene production, since once ACC was provided, it was readily converted to ethylene without dependence upon IAA (23). It is thus apparent that subsequent to the synthesis of methionine, the only auxin-regulated step in the biosynthesis of ethylene is the conversion of SAM to ACC.

Ethylene production and the conversion of methionine to ethylene are greatly inhibited by rhizobitoxine and its ethoxy analog, AVG, in a number of plant tissues (2, 10, 15, 19). In a previous study we have shown that the conversion of ACC to ethylene was insensitive to AVG inhibition (2, 23). Since AVG greatly inhibits conversion of methionine to ethylene, methionine to ACC, and auxin-dependent ACC accumulation (Table II), but does not affect the conversion of methionine to SAM (Table I), it is apparent that AVG inhibits the conversion of SAM to ACC, the same step upon which auxin exerts its inductive effect. This was further confirmed by the demonstration that AVG completely inhibited the enzymic conversion of SAM to ACC, the reaction catalyzed by ACC synthase (Table III). According to Boller *et al.* (3), the K_i value for AVG inhibition of ACC synthase isolated from tomato fruits was 0.2 μM.

Tracer studies have indicated that Co^{2+} inhibits ethylene production at a step or steps subsequent to methionine formation (14). In order to identify the step which is inhibited by Co^{2+}, we have compared the effect of various concentrations of Co^{2+} on the IAA-dependent conversion of SAM to ACC, and on the reaction in which ACC serves as the immediate precursor of ethylene (6, 23). As shown in Figure 1, Co^{2+} effectively inhibited both IAA-dependent ethylene production and ACC-dependent ethylene production. The inhibition was more pronounced in the later part of the incubation period in both systems, presumably due to the higher level of Co^{2+} accumulated. The concentrations of Co^{2+} required for 50% inhibition of ethylene production during a 6- to 9-h incubation period was estimated to be 20 μM for both systems (Fig. 2). Co^{2+} at 10 μM inhibited ethylene production in the IAA-dependent and ACC-dependent systems 20% and 10%, respectively. At 0.1 mM the inhibition was about 90% in both systems, and at 1 mM the inhibition was complete. Since both systems are inhibited by Co^{2+} in similar fashion, it may be concluded that Co^{2+} exerted its inhibitory effect at a common step, the conversion of ACC to ethylene. If this is the case, one may predict that endogenous ACC levels in IAA-treated tissues should increase as a result of the inhibition of ethylene production by Co^{2+} application. The results shown in Table IV are in full agreement with this prediction. In the presence of 50 μM Co^{2+}, ethylene production during a 6- to 9-h incubation period was inhibited 75%, but the ACC level at 9 h was 40% higher than that of control tissue. At a higher concentration of Co^{2+} (0.5 mM), ethylene production was further diminished while ACC accumulated. These results are in contrast to AVG-inhibited ethylene production, in which the ACC level decreased dramatically when IAA-induced ethylene production was inhibited by AVG.

Lau and Yang (14) have observed that Co^{2+} inhibited all ethylene production systems tested, including those of fruit tissues and vegetative tissues treated with IAA, kinetin, IAA plus kinetin, Ca^{2+}, kinetin plus Ca^{2+}, or Cu^{2+}. They have, therefore, suggested that Co^{2+} inhibited ethylene production by inhibiting an essential reaction common to all of the systems; we have now identified it as the conversion of ACC to ethylene, the last step in the biosynthetic sequence. The mode of action of Co^{2+} in inhibiting the conversion of ACC to ethylene is unknown. Except that the conversion of ACC to ethylene requires O_2 (2), little is known about the biochemical nature of this process. Co^{2+} is known to form a stable complex with sulfhydryl compounds (17). We have examined the effect of sulfhydryl reagents, *p*-chloromercuribenzoate, *N*-ethymaleimide, iodoacetate, and $HgCl_2$ on ACC-depend-

Table V. *Inhibition of ACC-dependent Ethylene Production by Sulfhydryl Reagents*

Each incubation flask contains 0.2 mM ACC and various sulfhydryl reagents at 0.1 mM. Ethylene production was determined during 6- to 9-h incubation period.

Inhibitor	Ethylene	
	nl	*% inhibition*
None	506	0
p-Chloromercuribenzoate	215	57
N-Ethylmaleimide	215	57
Iodoacetate	215	57
$HgCl_2$	184	64
$CoCl_2$	65	87

ent ethylene production by mung bean hypocotyls. At 10 μM these compounds exerted little inhibition (data not shown). However, at 100 μM ethylene production was inhibited more than 50% by each of these compounds as compared to 87% inhibition by Co^{2+} (Table V). These data suggest that Co^{2+} may inhibit ethylene production by interacting with the sulfhydryl group of the enzyme responsible for the oxidation of ACC to ethylene.

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